Identification of a Molecular Profile Associated with Immune Status in First-Onset Schizophrenia Patients

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Abstract

Objectives: Alterations in immunological parameters have been reported for schizophrenia although little is known about the effects of inflammatory status on immune-related functional changes at disease onset. Here, we have investigated such T cell-dependent molecular changes in first-onset, antipsychotic-naive schizophrenia patients using a novel *ex vivo* blood culture system. **Methods:** Blood samples from patients (n=17) and controls (n=17) were collected into stimulant-containing or null control TruCulture™ tubes, incubated 24 hours and the concentrations of 107 immune and metabolic molecules measured in the conditioned media using the HumanMAP™ immunoassay system. **Results:** Nine molecules showed altered release from schizophrenia blood cells compared to those from controls and this was replicated in an independent cohort. *In silico* pathway analysis showed that these molecules had roles in endothelial cell function, inflammation, acute phase response and fibrinolysis pathways. Importantly, five of these molecules showed altered release only after stimulation. **Conclusions:** This study has identified a reproducible peripheral molecular signature associated with altered immune function in first-onset schizophrenia subjects. This suggests that immune status can affect the biomarker profile which could be important for personalized medicine strategies. Furthermore, whole blood culture analysis may be useful in the identification of diagnostic tools or novel treatment strategies due to ease-of-use and clinical accessibility.

Key Words: Schizophrenia, Immune System, Blood Cells, Proteomics, Multiplex Immunoassay

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Introduction

There are numerous reports of immune abnormalities in the periphery and central nervous system of patients with schizophrenia (1-3). Cytokine levels have been measured in brain and body fluids such as cerebrospinal fluid (CSF) and blood serum of patients with schizophrenia and a decreased inflammation response has been linked with decreased production of T helper (Th)-1 cytokines (4). A previous study which carried out a meta-analysis has demonstrated alterations in cytokines in blood and CSF from patients with schizophrenia (5). However, some of these studies may be confounded by various factors such as differences in the number or type of cytokines measured (6, 7), disease subtypes, co-morbidities, illness duration or antipsychotic treatment. In addition, many of these studies have been per-

Clinical Implications

The authors have identified nine molecules that were secreted differentially from blood cells in *ex vivo* cultures obtained from first-onset patients with schizophrenia compared to control subjects. All of these analytes have been linked to schizophrenia before, although this is the first study which demonstrated the correlated nature of this response as a molecular signature associated with a pro-inflammatory status. Most of these molecules are involved in endothelial cell function and inflammation (20-22).

The authors used an *ex vivo* culturing system that required minimal sample handling and identified a reproducible peripheral signature comprised of immune and metabolic molecules. The correlation between these analytes further highlights their biological role in the inflammatory status which is manifested as altered expression of proteins associated with the acute phase response and the coagulation/fibrinolytic system. Most importantly, the results showed that immune status can affect the molecular profile, which has implications for future studies or clinical trials involving biomarker profiling. However, given the unavoidable use of low sample numbers, the current findings should be considered preliminary. Further studies using larger populations of first-onset patients with schizophrenia and controlling for immune status are warranted. This could lead to identification of novel personalized medicine strategies for patient stratification and more targeted treatment approaches.

formed using peripheral blood mononuclear cells (PBMCs), which may have led to inconsistencies related to different isolation procedures.

Cytokine responses are transient and interactive, and can be influenced by environmental factors such as cigarette smoking (8), antipsychotic medication (9) and illness in general (10). Therefore, it is important to measure the responses of multiple cytokines simultaneously to obtain a comprehensive picture of the functional effects. Moreover, measurement of other molecules such as hormones, lipids, proteases and transport proteins is important since these molecules are known to affect immune function and some of these have already been linked to schizophrenia. For example, increased levels of insulin have been reported in patients with schizophrenia and associated with increased prevalence of metabolic syndrome (11). Other studies have shown that peripheral lymphocytes of patients with schizophrenia have decreased expression of the receptor for reelin, a serine protease associated with schizophrenia pathology (12). In addition, membrane fatty acid abnormalities, including elevated levels of phospholipase A2 and impaired prostaglandin signaling, have been identified and linked to the reduced niacin skin flush response in schizophrenia patients (13).

With this in mind, we have investigated immune system function in first-onset, antipsychotic-naive schizophrenia subjects. The aim was to determine whether an inflammatory challenge at the onset of the disease could lead to a differential molecular profile. Blood was obtained from patients and controls and incubated with and without immune activation using a novel *ex vivo* system (TruCulture™), which more closely approximates *in vivo* conditions of immune cell activity compared to PBMC isolation procedures which are less likely to represent physiological conditions. This was followed by HumanMAP™ multiplexed measurement of approximately 100 immune and metabolic molecules released into the culture supernatants.

Materials and Methods

Study Population

The study was approved by the local ethics committees and conducted from 2008 to 2009 at the University Hospital of Cologne and at the Central Institute of Mental Health, Mannheim, Germany. Patients were diagnosed with paranoid schizophrenia *(DSM-IV*: 295.30) using the Structured Clinical Interview for the Diagnostic and Statistical Manual *(DSM-IV)*. Blood was collected on the day of first admission at the University Hospital of Cologne between 8 and 10 A.M. from fasted subjects comprising nine antipsychotic-naive (AN), first-episode subjects with paranoid schizophrenia and nine healthy control (HC) subjects with no family history of schizophrenia or detectable medical, psychiatric or neurological history. In addition, blood was also collected from an independent cohort at the Central Institute of Mental Health, Mannheim, comprising eight AN first-onset patients with paranoid schizophrenia and eight HC subjects for validation purposes (see Table 1). All patients and controls were matched using group means for the indicated demographic variables (see Table 1). There was no previous neurological history recorded for any of the patients used in this study. There was no significant difference in storage time of samples prior to analysis. It should be noted that AN subjects with schizophrenia are difficult to obtain as even large clinics see only 15–20 of these patients per year. HCs were matched for age, gender, smoking, cannabis use and

body mass index (BMI), and screened for medical disorders including psychiatric illness, autoimmune disease or recent infections. There were no differences in acute inflammation or infection conditions between the AN schizophrenia and HC groups as shown by the finding of no significant difference in the levels of C-reactive protein, which was measured as part of the multiplex immunoassay profiling analyses (see below). All AN patients with schizophrenia and HC subjects gave written informed consent for the study.

Table 1 Demographic Details

	Cohort 1			Cohort 2		
	HC	SZ	P-Value	HC	SZ	P-Value
Number (n)	9	9		8	8	
Age (years)*	30±8	29±10	0.979	29±6	30±7	0.465
Gender (male/female)	3/6	3/6	>0.999	3/5	3/5	>0.999
BMI*	23±3	24±7	0.836	25±4	22±2	0.395
Smoking (yes/no)	5/4	5/4	>0.999	5/3	5/3	>0.999
Cannabis (yes/no/na)	5/4	5/3/1	>0.999	6/2	3/5	0.315

*Mean±standard deviation. HC=healthy control; SZ=drug-naive, first-onset schizophrenia; na=not available; BMI=body mass index.
The p-values indicate that there were no significant differences between HC and SZ subjects in either of the two cohorts for any of the parameters measured.

TruCulture Sample Collection

Blood (4 mL) was collected into TruCulture tubes (Experimental & Diagnostic Immunology [EDI] GmbH Company, Germany). The TruCulture system allows for *ex vivo* culture of whole blood. This serves to eliminate artefacts introduced by separation and culture of blood cells using standard procedures and, therefore, allows for study of immune responses closer to the physiological conditions seen *in vivo*. The tubes contained either RPMI-1640 medium with no additional stimulant (unstimulated [US]) or RPMI-1640 medium supplemented with 1 µg/mL staphylococcus enterotoxin B (SEB [Sigma, Poole, UK]) and 1 µg/mL anti-CD28/CD49d (BD Biosciences, Oxford, UK) (stimulated [ST]) to target T cell responses with no effect on potentially interfering monocytes. One US and ST tube was collected for each subject, transferred to a thermo block and incubated at 37°C for 24 hours. Culture supernatants were separated from blood cells using an integral valve septum supplied with the tubes and stored at -80°C until analysis.

Multianalyte Profiling of Cell Supernatants

Culture supernatants of schizophrenia patients and HCs were analyzed using the HumanMAP™ multiplexed immunoassay platform at Rules Based Medicine (Austin, TX, USA) as described previously (14). These immunoassays target immune system, growth factor and hormonal molecules which have been implicated in many disorders including schizophrenia. Briefly, the 107 assays were calibrated using duplicate 8-point standard curves and raw intensity measurements were converted to absolute concentrations using proprietary software. Machine performance was verified using quality control samples at low, medium and high levels for each assay. Analyses were conducted under blind conditions with respect to sample identities and samples analyzed randomly to avoid any sequential bias due to factors such as diagnosis, participant age or time of storage of the material. Samples from the validation cohorts were measured subsequently using the same conditions. Expression levels of TGF-β1 were quantified using a commercially available enzyme-linked immunoabsorbent assay (ELISA) (R&D Systems, USA).

Statistical Analysis

Among all detected molecules, 11.2% and 12.5% of the data in the first and second dataset were missing due to typically encountered technical issues. These were assigned a value of 0.0. Data were analyzed after addition of a small constant (0.05) to all values and log-transformation. Each dataset featured readouts of identical samples from US and ST conditions. Repeated measures analysis of variance (ANOVA) was used to investigate the main effects and interactions of disease and stimulation. The difference between patients and controls in US samples was determined using analysis of covariance (ANCOVA). Diagnosis, age, sex, BMI, smoking and cannabis consumption were tested for covariance. $P<0.05$ was considered significant. Principal component analysis (PCA) was performed using SIMCA-P+ (Umetics, Umea, Sweden) to visualize the effect of stimulation on the subjects, based on differences in levels of molecules. To ensure uniform weighting, all molecular values were scaled prior to PCA to have a mean of 0 and a standard deviation of 1.

P-values were corrected for multiple hypothesis testing by determining the False Discovery Rate (FDR) according to Benjamini and Hochberg (15). The advantage of this method is that the stringency of the adjustment is dependent on the overall number of significant findings. For the present study, FDR control was chosen since the more conservative control of the family-wise error rate would lead to an unnecessary rejection of potentially interesting biomarker candidates

Correlation and Ingenuity Pathway Analysis Networks

Pearson correlation analysis of molecules was performed using Prism v5.0 (GraphPad Software, La Jolla, CA, USA). Correlations were calculated for all possible analyte combi-

nations and considered significant for $r>\pm0.4$ and $p<0.05$. Correlation networks were generated based on significant interactions between molecules. Ingenuity Pathway Analysis networks were generated using the Ingenuity Pathway Knowledgebase (IPKB) software as described previously (16).

Results

Assessment of Data Quality

TruCulture tubes containing medium with or without stimulant were used to investigate differential immune responses in blood samples from two independent cohorts of AN schizophrenia patients and HCs (see Table 1). Principle component analysis (PCA) was used in independent testing of the two cohorts to assess the effect of stimulation on the distribution of subjects, based on differences in culture supernatant concentrations of molecules. Distinct separations were seen between the US and ST conditions for both cohorts (see Figure 1).

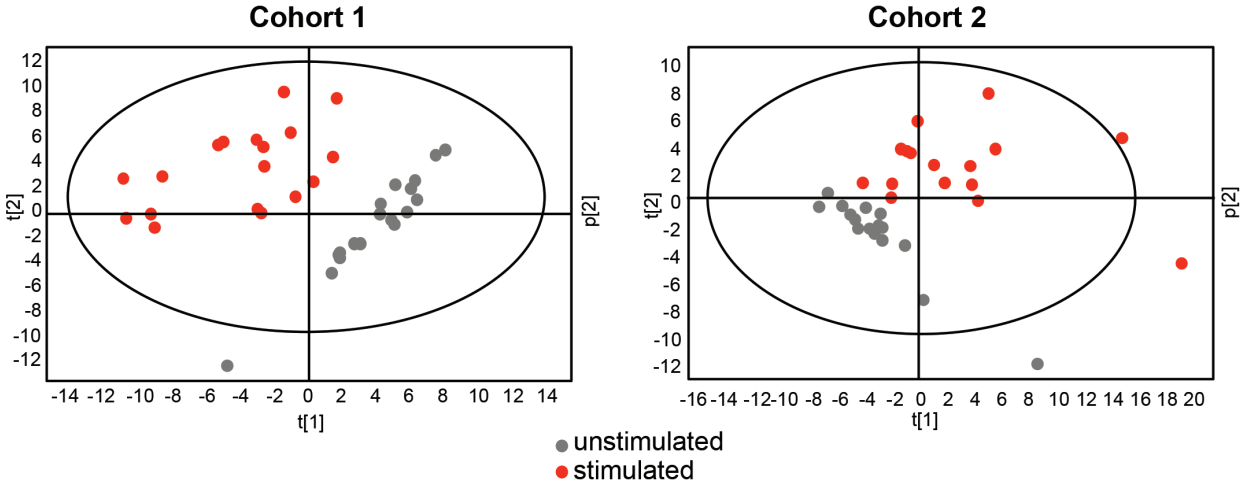
Effects on Unstimulated Cells

Differentially expressed molecules in culture supernatants of US TruCulture incubations were identified initially for cohort 1 using ANCOVA. The molecules were stratified for underlying baseline characteristics including cannabis use, smoking, age, gender and BMI which can affect immune-related parameters (17, 18). Nine molecules were differentially expressed ($p<0.05$) between AN patients with schizophrenia and HC subjects (see Table 2A). To validate these findings, the same statistical method was applied to the second cohort, which identified 22 differentially expressed molecules (data not shown). Four analytes showed decreased expression in both cohorts: matrix metalloproteinase 3 (MMP3), angiotensin converting enzyme (ACE), α-2 macroglobulin (A2M) and thyroid stimulating hormone (TSH) (see Table 2A).

Effects on Stimulated Cells

It was also of interest to investigate whether stimulation evoked a different response between AN patients with schizophrenia and HC subjects, and whether this was reflected by differential release of molecules into culture supernatants. Eleven molecules were identified in cohort 1 that fulfilled these criteria (see Table 2B). Of these, five (progesterone, IL-18, ENRAGE, plasminogen activator inhibitor 1 [PAI-1] and myeloperoxidase) also showed differential release in supernatants from the second cohort (see Table 2B). Note that p-values indicate whether the interaction between diagnosis and the stimulation condition was significant. Therefore, molecules can be differentially expressed between patients and HCs in either of the stimulation conditions but not necessarily in both. For example, ENRAGE, progesterone and PAI-1 showed a difference in expression levels for the US condition, whereas the expression levels of these three analytes were equal between patients and HCs in the ST condition. The observed changes in expression were analyzed further using stimulation indices (SI, ratio ST/US) for the most reproducible findings (see Figure 2). The SI of myeloperoxidase, IL-18 and ENRAGE was positive for patients and HCs upon stimulation. In contrast, expression levels of progesterone and PAI-1 changed in opposite directions between patients and HCs after stimulation.

Figure 1 Data Quality



Independent principle component analyses showing the separation between unstimulated (grey) and stimulated (red) samples for cohort 1 (left) and cohort 2 (right) based on the levels of molecules. The ellipse indicates the boundaries of the 95% confidence interval.

Table 2A Altered Analytes in Schizophrenia Patients Irrespective of Stimulation Condition

Acc	Analyte	P-Value*		Fold Change (SZ/HC)	Rep
		Cohort 1	Cohort 2		
P06732	Creatine Kinase M/B	0.009 (0.406)	0.360	-2.3	x
P08254	MMP 3	0.019 (0.406)	0.008	-1.4	✓
Q8CFN1	ACE	0.024 (0.406)	0.057	-1.5	✓
N/A	Cortisol	0.034 (0.406)	0.158	1.3	x
P05543	TBG	0.038 (0.406)	0.411	1.1	x
P01023	α-2 macroglobulin	0.040 (0.406)	0.027	-1.1	✓
P40225	Thrombopoietin	0.044 (0.406)	0.467	-1.2	x
P01215	TSH	0.045 (0.406)	<0.001	-1.6	✓
P05362	ICAM 1	0.045 (0.406)	0.286	-1.2	x

*ANCOVA for unstimulated TruCulture™ systems. False discovery rates (q-values) were also calculated for cohort 1. Acc=Swiss-Prot accession; SZ=antipsychotic-naive schizophrenia; HC=healthy control; Rep=reproducibility across cohort; (✓)=reproducible and (x)=not reproducible; MMP=matrix metallopeptidase; ACE=angiotensin converting enzyme; TBG=thyroxine binding globulin; TSH=thyroid stimulating hormone; ICAM=inter-cellular adhesion molecule.

Table 2B Altered Analytes in Schizophrenia Patients Depending on Stimulation Condition

Acc	Name	P-Value*		Fold Change (SZ/HC)		Rep
		Cohort 1	Cohort 2	US	ST	
P05231	IL-6	0.013 (0.375)	0.989	3.5	-1.1	x
N/A	Progesterone	0.016 (0.375)	0.086	1.3	1.0	✓*
Q14116	IL-18	0.020 (0.375)	0.006	-1.4	-1.1	✓
P80511	ENRAGE	0.021 (0.375)	0.020	1.5	1.0	✓
P05121	PAI-1	0.023 (0.375)	0.077	-1.2	1.0	✓*
P01133	EGF	0.030 (0.375)	0.217	-1.2	1.0	x
P41159	Leptin	0.031 (0.375)	0.217	-1.1	1.2	x
P05164	Myeloperoxidase	0.034 (0.375)	0.068	1.2	-1.1	✓*
P40225	Thrombopoietin	0.035 (0.375)	0.525	-1.2	1.0	x
P01015	Angiotensinogen	0.048 (0.432)	0.347	-1.3	-1.8	x
P21583	Stem Cell Factor	0.048 (0.432)	0.384	-1.2	1.2	x

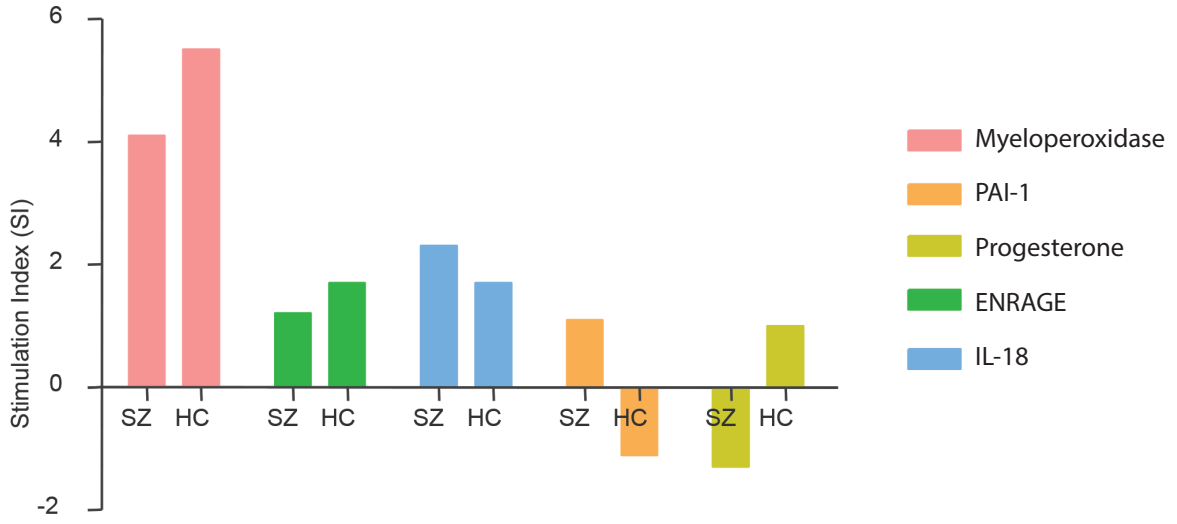
repeated measures ANOVA calculating significant interaction between diagnosis (diseased/healthy) and stimulation condition (unstimulated/stimulated). False discovery rates (q-values) were also calculated for cohort 1. Additional abbreviations: (✓)=reproducible with borderline significance; IL=interleukin; PAI=plasminogen activator inhibitor; EGF=epidermal growth factor.

Statistical and Pathway Analyses

Pearson correlation analyses were employed to assess whether expression levels of the nine reproducible analytes were correlated as a means of further validating the results (see Figure 3A). All analytes were tested in combination because molecular changes can be linked intermittently and could have effects on the immune response in either US or ST conditions. Significant interactions were found for A2M, which correlated positively ($r>0.4$, $p<0.05$) with PAI-1 and ACE, and negatively with MMP3 ($r>-0.4$, $p<0.05$). PAI-1 and ACE were also correlated with IL-18 and TSH. These six analytes formed a single correlation network and also mapped to the highest scoring IPKB network with associated functions indicated as "protein degradation" and "cardiovascular system development and function" (see Figure 3B). ENRAGE and myeloperoxidase (MYO) formed a separate correlation network which also mapped to a unique IPKB network with the associated functions "antigen presentation" and "cell-mediated and humoral immune response" (see Figure 3B, right). Progesterone did not correlate with any other analytes. As all patients were acutely psychotic at the time of blood withdrawal, cortisol levels were also measured in culture supernatants as a potential indicator of stress. Although cortisol levels were significantly different between patients and controls in cohort 1, the effect was not reproduced in cohort 2. Also, no correlations between cortisol and any other analytes in US or ST samples from patients and controls were identified (data not shown), suggesting that stress had no impact on secretion of these molecules.

The two most significant canonical pathways associated with the nine differentially expressed analytes were "acute phase response" (A2M, IL-18 and PAI-1, p=9.2E-05) and "coagulation/fibrinolytic system" (A2M and PAI-1, p=1.72E-04). Interestingly, both pathways were connected through five out of the nine differentially expressed molecules and all of these were significantly decreased in patients compared to HCs (see Figure 4). TGF-β was identified as a key component within the network, exerting an inhibitory role on MMP-3 and a stimulatory role on PAI-1 and A2M (19). As TGF-β was not represented on the HumanMAP panel, we measured the expression levels of this molecule separately using a commercially available ELISA kit. This showed that there was no difference in TGF-β release between patients and controls for either the US or ST conditions. It should be noted that the assay used would not nec-

Figure 2 Stimulation of Differentially Expressed Analytes



The stimulation index of the most reproducible differentially expressed analytes between schizophrenia patients (SZ) and healthy controls (HC) was determined as the ratio of measured analyte concentrations in the stimulated compared to the unstimulated condition.

essarily distinguish between the precursor form of TGF-β (proTGF-β) and the mature active form.

Discussion

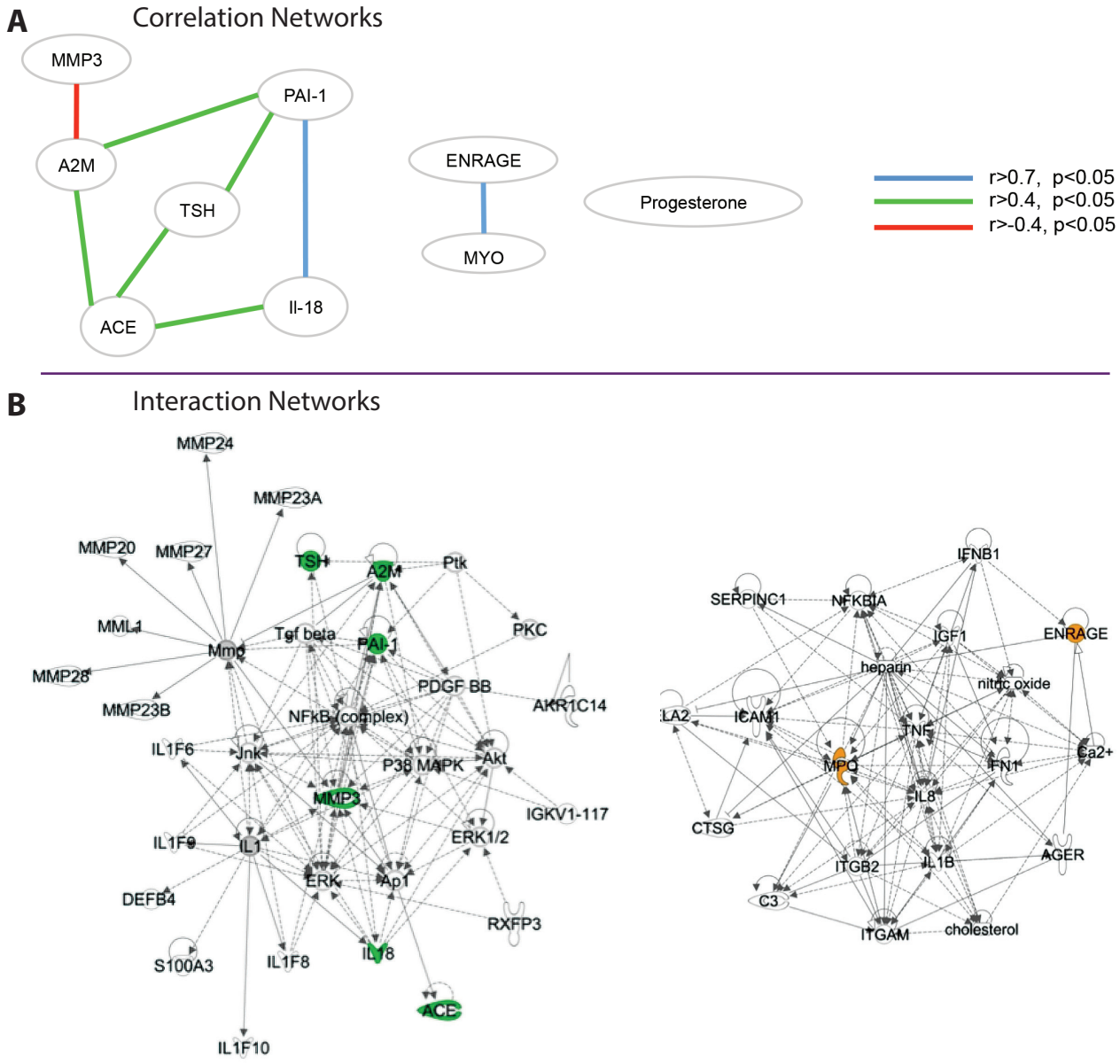
We have identified nine molecules that were secreted differentially from blood cells in *ex vivo* cultures obtained from first-onset patients with schizophrenia compared to control subjects. All of these analytes have been linked to schizophrenia before, although this is the first study which demonstrated the correlated nature of this response as a molecular signature associated with a pro-inflammatory status. Most of these molecules are involved in endothelial cell function and inflammation (20-22).

Given that fibrinogen is a component of the acute phase response and the coagulation/fibrinolytic system, it might at first seem surprising that we did not identify this as an altered molecule in this study. However, fibrinogen is a major component of clots formed in the preparation of serum, which we have used in this study. Therefore, it is likely that most of the fibrinogen would have been unavailable for analysis.

The endothelial system contributes to activation of the acute phase response (APR), which stimulates the coagulation/fibrinolytic system and affects vascular permeability (23). This process is also tightly regulated through ACE, which was decreased in patients and could, therefore, lead to altered levels of vasoactive molecules such as angiotensin II and bradykinin, with effects on leukocyte migration, sensitivity to pain and vascular permeability (24). This is consistent with the well-known reduced niacin skin flush response of patients with schizophrenia (25) and could also be explained by increased levels of MYO which would affect nitric oxide production by endothelial cells (26).

Decreased levels of TSH have previously been associated with endothelial dysfunction and are a common feature of clinical hyperthyroidism (27). As TSH, PAI-1 and A2M inhibit the coagulation/fibrinolytic system (28), reduced expression of these molecules in patients with schizophrenia would be predicted to lead to an increase in vascular permeability. Under inflammatory conditions, vascular permeability is increased in order to promote leukocyte trafficking to the site of inflammation (29). Although levels of positive APR proteins are also increased under inflammatory conditions (30), we found that IL-18, A2M and PAI-1 were decreased in the unstimulated condition in patients compared to controls. This suggests the possibility of an immune system dysfunction in schizophrenia patients associated with an impaired inflammatory response (19). PAI-1 regulates the coagulation/fibrinolytic cascade through inactivation of tissue plasminogen activator (tPA) (19). This leads to inactivation of plasmin which normally converts TGF-β and MMP3 to their active forms. We found that release of MMP3 was decreased, although that of TGF-β was similar between patients and controls. However, the immunoassay used to measure TGF-β does not distinguish between the TGF-β precursor and the proteolytically cleaved active form of the molecule. Therefore, we cannot establish whether secretion of active TGF-β was affected. Also, it should be noted that the molecules tested by the multiplex immunoassay system in this study are not completely representative of the

Figure 3 Correlation and Interaction Networks

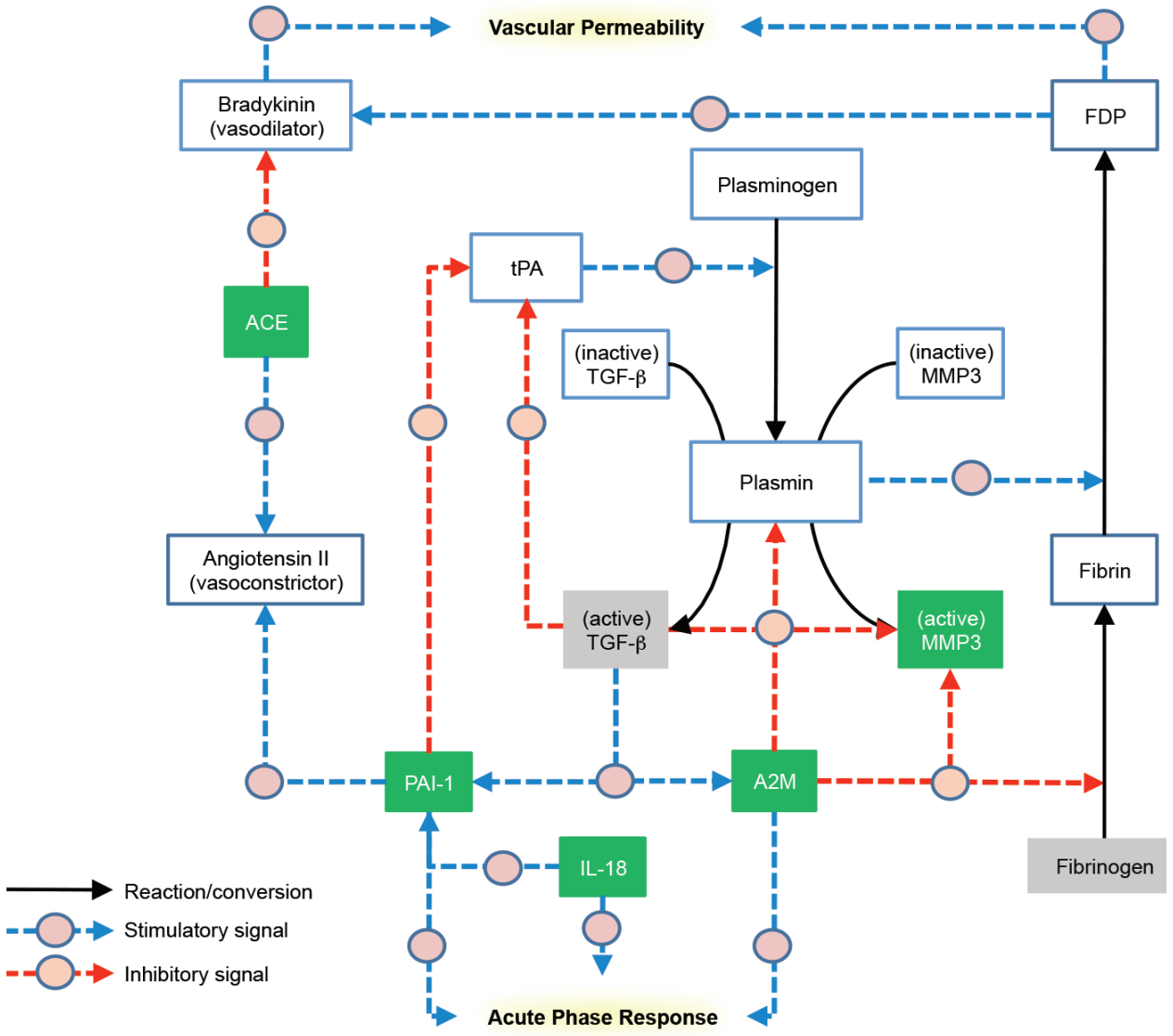


(A) Correlation networks for the 9 reproducible differentially expressed analytes between schizophrenia patients and healthy controls. Correlations (colored lines) between analytes were considered significant if they had a Pearson correlation coefficient of r<0.4, r<-0.4 and a p-value of p<0.05. **(B)** Ingenuity Pathway Knowledgebase (IPKB) networks of the 9 analytes (green and orange in the left and right networks, respectively). Non-colored interacting analytes were added by the software according to known associations in the scientific literature.

pathways targeted, as this is dependent upon the available panel of antibodies in the platform. It is, therefore, possible that other molecules from the same and other physiological pathways are also affected. In addition, this study has focused on stimulation of specific T cell populations through the use of SEB and anti-CD28/49. Future studies should incorporate the use of reagents which stimulate different classes of T cells for a more comprehensive picture of the effects.

One key advantage of the present study is that we have analyzed unique sets of samples which are difficult to obtain (i.e., antipsychotic-naive, first-onset patients with schizophrenia and well-matched control cohorts) to minimize potential confounding effects. This naturally restricted the number of subjects that could be used, although we selected only those biomarkers which were reproducible in two independent cohorts. However, it should be noted that the impact of these studies is limited by the small sample size.

Figure 4 Scheme Showing the Relationship between Whole Blood Culture Supernatant Molecules and Involvement in the Coagulation System and Acute Phase Response



Molecules were decreased (green), unchanged (grey) or not measured (white) in patients compared to controls. Plasmin promotes fibrinolysis which results in increased release of fibrinogen degradation products (FDP). Plasmin is inhibited by plasmin activator inhibitor 1 (PAI-1) via inactivation of tissue plasminogen activator (tPA), which promotes formation of plasmin from plasminogen. Alpha-2 macroglobulin (A2M) exerts antifibrinogenic actions through inhibition of plasmin, active matrix metalloproteases (MMP) and thrombin. The active forms of MMP3 and transforming growth factor-β (TGF-β) are generated by plasmin. TGF-β plays dual roles as an inhibitor of tPA and MMP3 and as an activator of PAI and A2M. PAI-1, A2M and IL-18 are components of the acute phase response. PAI-1 and angiotensin converting enzyme (ACE) are potent inducers of vasoconstrictors. ACE further degrades bradykinin, a vasodilator that increases together with FDP vascular permeability.

Therefore, it is important to repeat these studies using additional and larger cohorts. Also, one problem in constructing a molecular test from the present findings is that inflammation and vascular abnormalities are not limited to schizophrenia and are found in numerous other disorders. In addition, there is compelling evidence that psychosis is linked to psychosocial stressors and that schizophrenia patients respond more strongly to stress compared to healthy subjects (31). However, we ruled out differential stress effects by showing that cortisol levels, measured as part of the multiplex immunoassay platform, were not altered reproducibly in culture supernatants of schizophrenia patients compared

to controls. Moreover, there were no significant correlations between the levels of cortisol and those of other analytes.

In conclusion, we used an *ex vivo* culturing system that required minimal sample handling and identified a reproducible peripheral signature comprised of immune and metabolic molecules. The correlation between these analytes further highlights their biological role in the inflammatory status which is manifested as altered expression of proteins associated with the APR and the coagulation/fibrinolytic system. Most importantly, the results showed that immune status can affect the molecular profile, which has implications for future studies or clinical trials involving biomarker profiling. However, given the unavoidable use of low sample numbers, the current findings should be considered preliminary. Further studies using larger populations of first-onset patients with schizophrenia and controlling for immune status are warranted. This could lead to identification of novel personalized medicine strategies for patient stratification and more targeted treatment approaches.

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Statement of Interest

Authors HR, ES and PCG are consultants for Rules Based Medicine.

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